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During the past year our project has evolved dramatically and significant progress has been made with respect to our overall goals of defining the genetic changes that distinguish normal cells from tumor cells of the breast. The most important evolution of the project is our change in strategy from identifying presumed tumor suppressor genes defined by loss of heterozygosity (LOH) using a positional cloning approach, to ascertaining and comparing genetic expression profiles from surgical specimens or cells grown in tissue culture using hybridization of cDNA from our tissues to microarrays of interesting genes. This is a very new technology and we are using a novel micro arraying and scanning instrument recently acquired by our laboratory in collaboration with Molecular Dynamics/Amersham. Preliminary experiments suggest that the sensitivity of our new microarray system is sufficiently high to allow us to detect variance in gene expression levels with high precision even under complex hybridization conditions. We have also applied the Differential Display protocol to identify three genes with altered expression in a model system of normal and neoplastic epithelial colonocytes. As an alternative to determining biologic effects of specific genes, we have tested and selected an amphoteric retroviral conditional expression system, which replaces previous experiments using antisense oligo's. We are confident that the "modernization" of our research project will facilitate the identification of genes critical to the development and progression of breast cancer.

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Ray White 8/14/97

PI - Signature Date

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Introduction

The overall goal of this project is to identify and characterize breast cancer genes. Until now the emphasis of the project has been directed towards the identification and characterization of several specific tumor suppressor genes located on chromosome 17. Common for each of these genes was that their approximate physical location had been delineated either through genetic linkage analysis of hereditary segregation patterns or by being the target of recurring deleterious events observed as loss of heterozygosity (LOH) in sporadic tumors. Since the discovery of the BRCA1 gene responsible for a hereditary cancer syndrome our experimental focus have been directed towards the identification of four presumed tumor suppressor genes defined by LOH on chromosome 17. Of these four loci, two are located at each extremity of the chromosome, while the remaining two areas of LOH we have identified flank either side of the BRCA1 locus at 17q21. Contrary to hereditary tumor suppressor genes who's position may be determined by genetic linkage analysis, the localization of tumor suppressor genes by LOH is circumstantial. Therefore, LOH does not provide direct evidence as to whether the region of LOH actually contains a gene critical to carcinogenesis or whether the deletion is coincidental. Statistically significant areas of LOH indicative of a tumor suppressor gene can only be determined by analyzing large numbers of specimens. At the same time it holds true that coincidental deletions provide grounds for mis-interpretation of the position of an actual tumor suppressor gene. When our proposal was submitted this paradigm represented the most efficient approach to identifying tumor suppressor genes, however, it has become exceedingly clear that the massive resources applied in various genome centers to establish both physical and expression maps covering the entire human genome far exceeds the amount of data that were are able to generate even within the modest areas of chromosome 17 where we have concentrated our attention. The past year has been a period of transition for the project and early on during this period we evaluated the efficiency of our strategy compared to newer, more global strategies to identify genes critically involved with tumor development and progression. We have for a long time realized that LOH guided search for tumor suppressor genes is vulnerable to critique on several levels. Most importantly among these the rather poor delineation of the tumor suppressor loci, which consequently requires a significant expansion of the physical mapping component of the project and thus dramatically augment the number of candidate genes to evaluate. A different and much broader strategy for identifying *any* kind of gene involved in tumor formation and progression, which is not dependent on prior knowledge of location, employs genetic profiling using high density micro arrays.

Not only does this technique permit detection of the expression profiles of each of a large number of genes in parallel, but it potentially also provides a mechanistic view of how regulatory pathways are controlled. As an entry to this technology we have established a collaboration with Molecular Dynamics who has provided us with an array robot and a scanning device.

Body

During the past 12 months we have made progress in several areas relating to our research proposal and have also explored new research avenues. In the following sections we will describe in detail the current status of our project. With respect to Task 1, which focuses on "Isolation and characterization of two potential tumor suppressor genes approximately 1MB proximal and 1 MB distal of BRCA1", we have extended the cDNA sequence of the FKBP65 gene to over 2 kb. By Northern analysis the gene product is predicted to be encoded by a 2.4 kb message, similar to the size of the murine homologue of FKBP65 (Coss et al. 1995). Unfortunately, RACE experiments to obtain the five-prime extremity of the gene have proven unsuccessful as well as the general sequencing of the gene has proven surprisingly difficult due to several internally repeated DNA segments complicating the sequencing of PCR amplified DNA templates. Within the known sequence, we have determined the location of several exon boundaries by comparing genomic and cDNA sequences. In addition hereto, we have evidence indicating that the gene transcript exists in two or more abundant splice forms. During the sequence analysis of a genomic clone close to the human FKBP65 gene we noted the presence of on unrelated expressed DNA segment with high sequence homology to a hypothetical C. elegans gene. Only one additional human EST showing similarity to this gene has been recorded in the public sequence database, and that gene has been characterized as a novel interferon-α-induced protein associated with Lupus inclusion known as p36 (Rich et al. 1996; Vakharia et al. 1996) . We expect to report the completion of the full-length sequence of both of these genes soon.

Because of the negative results we have obtained in our experiments to measure the growth effects of three cultured cell lines following the addition of DLG2 and DLG3 antisense oligonucleotides to the growth medium (described in our previous report), we have found it necessary to implement a more sensitive, reliable and conditional gene expression system to better measure the biologic effects of the genes we identify as candidate tumor suppressor genes. We have therefore acquired four amphotropic retroviral vectors which allow for conditional suppression (or expression) of cloned genes. To determine the biologic characteristics of the first of these vectors, LINX (Hoshimaru et al. 1996), we introduced the Green Fluorescent Protein (GFP) into the vector and infected three different breast cancer cell lines with the construct. The results of these experiments, summarized in Table 1, indicate that the LINX vector can be infected into breast cancer cells with efficiencies in excess of 75%. They also indicate that while GFP is a highly stable protein

the overall concentration steadily declines to minimal levels over a period of four to eight days following transcriptional inhibition by the tetracycline analog, doxycyclin (DOX). We are convinced that the change to this effective method for testing the biologic effects of individual genes will provide clearer and more convincing data than the effects of antisense oligo's, and we are in the process of obtaining full-length constructs of both of the DLG genes to test their biologic effects. Please note that the results reported in the table below apply to the induction and repression of our GFP marker in mass culture. We anticipate that we will be able to obtain individual clones that show better levels both of expression and repression.

Breast Cancer Cell line	Cell-type specific autofluorescence with LINX vector alone	% Cells fluorescent following LINX-GFP infection	Mean Fluorescence Intensity following LINX-GFP infection	Mean Fluorescence Intensity 4 Days after DOX addition	Mean Fluorescence Intensity 8 Days after DOX addition	Residual GFP after eight days of incubation with DOX
MCF7	1.65	76.04	228	90	15	6.6%
BT549	2.02	85.32	629	294	28	4.5%
MDA-MB468	1.82	76.86	599	323	56	9.3%

Table 1. **Doxycyclin (DOX) repression of LINX GFP in infected breast cancer lines as determined by FACS analysis.**

To test the efficacy of the tetracycline repressible LINX vector system, GFP was cloned into the LINX vector. Show here are the results of the addition of DOX to LINX-GFP infected breast cancer cell lines. The effects of DOX suppression is clearly visible after four days of incubation and after eight days incubation, the fluorescent signal is reduced to a small percentage of the original. The GFP signal detected at the eight day mark could be caused either by incomplete suppression of the Tet repressor, alternatively it could be argued that the signal is residual and results from the slow turn-over rate of the highly stable GFP protein.

Our initial strategy for identifying tumor suppressor genes in areas of LOH on chromosome 17 was based on a positional cloning strategy. This strategy is divided into three separate stages.

During the first stage a physical map of a the area of interest is constructed using genomic clones like YACs, BACs or P1s. Following this stage expressed sequences are ascertained, and as the last stage the candidate genes are analyzed for mutations in breast tumors to determine if indeed they are the sought-for tumor suppressor gene. While we successfully have used this approach in the past (Cawthon et al. 1990; Groden et al. 1991; Joslyn et al. 1991; Viskochil et al. 1990), we recognize that this strategy is both labor intensive and only moderately efficient. The inherent inadequacies of the strategy have been further accentuated by the efforts of some genome centers to physically map the entire genome (Cohen et al. 1993) and to position expressed sequences along each chromosome (see below), which have resulted in a very extensive overlap with our efforts. We have encountered numerous cases hereof, but one typical example of this overlap is that expressed sequence tags (EST's) corresponding to both of our newly discovered genes in the plakoglobin region, FKBP65 gene and the C. elegans homolog, independently were identified and localized between the genetic markers D17S798 and D17S800 – exactly in the area of plakoglobin. To document this situation, a schematic representation of the known and unknown genes genetically or physically mapped to specific regions of chromosome 17 accessible on the web (www.ncbi.nlm.gov/SCIENCE96/) have been included in the appendix. From this list it can be determined that in the area of chromosome 17pter alone, - the locale of the tumor suppressor genes we currently are searching for -, a total of 166 unique cDNAs have been mapped. This situation has been difficult for us to continue and has required us to redefine the means and methods necessary to achieve the original objective of our project; *to identify and characterize genes involved in breast cancer development and progression.* Having evaluated the situation, we determined that we would have to choose one of the following two experimental paths: a) determining the full-length sequence of previously mapped genes and screening them for mutations in breast tumors, or b) implementing a novel cancer gene identification strategy with a wide scope and of high efficiency. We chose to follow the latter path and in the following sections we describe the two approaches we have implemented to identify differentially expressed genes and to determine genetic expression profiles.

As our initial experimental model we decided to compare the expression patterns of normal and neoplastic cells by the *Differential Display* method (Liang and Pardee 1992) to identify genetic alterations characteristic of the early stages of tumor progression. The problem of adapting this approach to studying epithelial breast cells is that neither normal nor neoplastic epithelial breast cells

can be obtained as a homogenous population in sufficient quantities from surgical specimens. As an

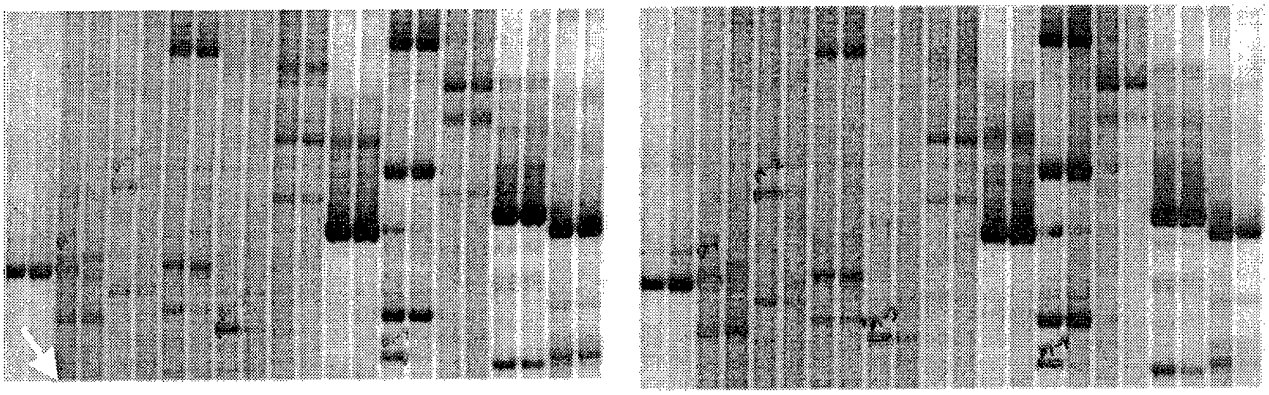


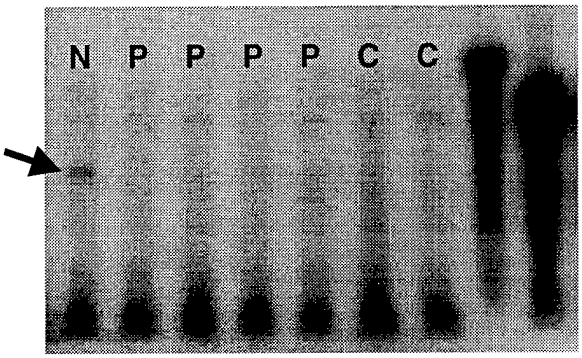
Figure 1. **Differential Display Gel.**
This figure shows eleven paired normal and polyp samples tested with the Differential Display system. An RT-PCR product marked by the white arrow was recovered from the gel and identified as the differentially expressed gene CGM2. Also note the high degree of the high level of identity between paired lanes and between the two independent PCR preparations represented in the left and right panels.

alternative to the breast epithelial cells we selected to compare purified normal colonic crypt cells to crypt cells isolated from adenomatous colonic polyps. While the biologic function of breast and colonic epithelial cells differ from each other, the rationale for using colonic crypt cells is firstly based on the fact that the human colonic adenomas are deficient in the APC tumor suppressor protein, which has been shown in mouse to be predisposing for mammary carcinoma as well as colon carcinoma (Moser et al. 1995; Moser et al. 1993). Furthermore, many human mammary tumors have been shown to be deficient in E-cadherin (Kanai et al. 1994; Rimm et al. 1995; Schmutzler et al. 1996), an important cell adhesion protein whose activity is modulated by APC and b-catenin, each of which are members of the Wnt regulatory pathway (Tao et al. 1996; Vleminckx et al. 1997). It is also worth noting that Wnt-1 is a gene first identified as the target gene for mouse mammary tumor virus in MMTV-induced mouse mammary tumors. As our experimental procedure, we prepared first strand cDNA from total RNA isolated from either normal or adenomatous crypt cells using a collection of twelve anchored poly(T) down-stream primers. Each of the first strand cDNAs thus synthesized subsequently served as template in a PCR reaction against twenty different arbitrarily designed up-stream primers. A total of 240 sample-pairs were prepared in this way and the resulting radiolabeled PCR products visualized in acrylamide gels. To confirm reproducibility of the PCR band patterns all samples were prepared and tested in duplicate (Fig.1). Our Differential Display comparison of the normal and adenomatous colonic crypt cells has been completed, resulting in the

identification of at least three genes (CGM2, prothymosin α, and a novel gene with similarity to X box binding protein-1) which consistently and reproducibly show altered expression levels in a panel of two normal and six polyps (Fig. 2) (Albertsen et al. - manuscript in preparation). In addition to these three genes more than 30 genes reproducibly displayed altered expression levels in the Differential Display gels, however the very low abundance of these messages in the tissue samples we analyzed prevented us from confirming their exact expression status in traditional methods like Northern Blot analysis and RNase Protection Assay.

Several hybridization based methodologies to quantitatively measure expression levels of large numbers of arrayed genes has been described during the past years ((Fodor et al. 1993; Pease et al. 1994; Schena et al. 1995). While the concept and biochemical principles of this type of analysis are simple, the implementation requires sophisticated and costly robotics and laser-scanning technology not readily available. Therefore, to gain access to this technology our laboratory has established a

Figure 2. **RNase Protection Assay of CGM2.**
To confirm the differential expression level of genes identified using the Differential Display system, we compared the expression levels from normal crypt cells (N), adenomatous polyp cells (P) and carcinoma cells (C). A band indicating that the CGM2 gene is expressed only in normal crypt cells and not in the polyp or cancer cells is indicated by the black arrow.



collaboration with Molecular Dynamics, a company which has developed a full set of instrumentation for creating and reading microarrays. We are now using our instruments to analyze gene expression in various breast cells to identify genes displaying altered expression levels among epithelial breast cell lines. As our initial collection of genes to analyze we have used chosen the genes identified using Differential Display on colonocytes described above. In initial experiments, we have been able to confirm that the three transcripts that vary in the normal vs. adnomatous colonocyte also vary in a normal by carcinoma breast cell comparison. Following these initial experiments we will extend the array to include our entire panel of more that 1500 unique cDNA clones selected to represent various classes of biochemical function likely to be relevant to breast carcinogenesis.

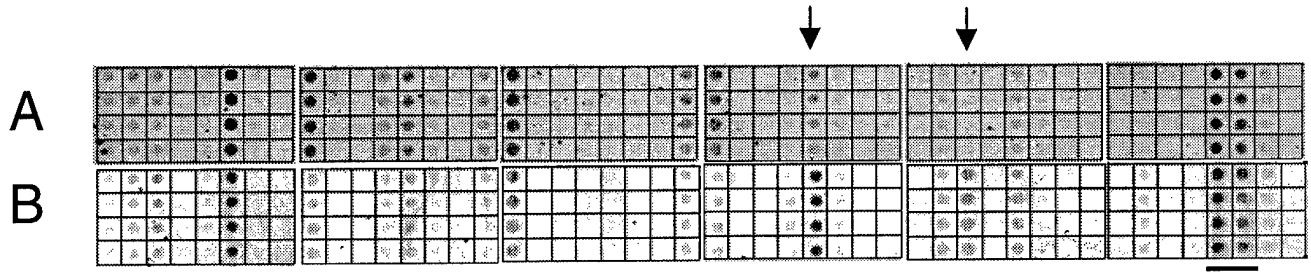


Figure 3. Microarray Analysis of breast cells BE45 and MDA-MD486.
Panel A is a fluorescent scan of a microarray which was hybridized to fluorescently labeled first strand cDNA derived from the mRNA of the normal breast cells BE45. Panel B is an identical array, which was hybridized to fluorescent cDNA derived from breast cancer cells MDA-MD486. The microarrays contain 47 different cDNA clones and a plasmid (pBS) control. Each column contains a different clone which was spotted four times. The arrows point to two clones, prothymosin-α and a homolog of the X box binding protein 1, which reveal different expression levels of the two genes between the two cell types. The two strongest hybridizing clones underlined on the right side of the panel are the house keeping genes Cyclophilin and GAPDH.

In light of our recent acquisition of a Microarray Spotting and Scanning instrument and the possibility of using the microarray technology to establish genetic expression profiles from any cell source of interest, the latter alternative stands out as having the highest scientific potential. We therefore propose to apply the microarray scanning technology to establish genetic profiles from the experimental model system describing the reversion of the malignant phenotype of breast cells developed by Dr. Weaver (Weaver et al. 1996; Weaver et al. 1997).

Conclusions

This past year has been a period of transition for the project. New avenues have been explored and as a consequence we recognize that we have not produced as many specific results as in previous years. Nevertheless, we firmly believe that a change in methodology was necessary and we are convinced that the adjustment period will be generously compensated by the insight we expect to gain from the application of micro arrays to determine genetic expression profiles in normal and cancerous epithelial breast cells.

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Appendix

Map Search

Chromosome
17

Go to:
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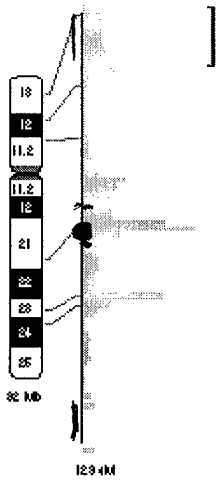
6 7 8 9 10
11 12 13 14 15
16 17 18 19 20
21 22 X



Search Interval

Enter the names of the Genethon markers which define the desired search interval.

Select Markers

Interval: pTel-D17S960

166 cDNA markers in this interval

Between 17pter and D17S849 [< 0 cM]

A003A09	Unidentified transcript

Near D17S849 [0 cM]

BCD2293	Unidentified transcript
IB500	Unidentified transcript
SGC34337	Unidentified transcript
WI-7998	Unidentified transcript

Between D17S849 and D17S796 [0-14 cM]

S67156	ASPARTOACYLASE
stSG9972	DPH2L=candidate tumor suppressor gene {ovarian cancer critical region of deletion} [human, 9 week fetal and placental tissues, mRNA, 2233 nt]
A005T43	EUKARYOTIC INITIATION FACTOR 4A-I
Cda0gb02	H.sapiens alternative 3' UTR of Nup88 mRNA
WI-6584	H.sapiens alternative 3' UTR of Nup88 mRNA
SGC35667	H.sapiens mRNA for RABAPTIN-5 protein

stSG4272	H.sapiens mRNA for adenosine triphosphatase, calcium
WI-11146	Highly similar to ARRESTIN-C [Rattus norvegicus]
stSG9838	Highly similar to ARRESTIN-C [Rattus norvegicus]
stSG8413	Highly similar to BETA ENOLASE [Homo sapiens]
A008L05	Highly similar to HYPOTHETICAL 13.5 KD PROTEIN C45G9.7 IN CHROMOSOME III [Caenorhabditis elegans]
BCD2371	Highly similar to HYPOTHETICAL 13.6 KD PROTEIN IN NUP170-ILS1 INTERGENIC REGION [Saccharomyces cerevisiae]
A002D39	Highly similar to HYPOTHETICAL 264.2 KD PROTEIN IN RAD3-BMH1 INTERGENIC REGION [Saccharomyces cerevisiae]
A006R35	Highly similar to MEIOSIS-SPECIFIC SPORULATION PROTEIN SPO14 [Saccharomyces cerevisiae]
SGC35694	Highly similar to THIOREDOXIN [Homo sapiens]
A006J03	Highly similar to ZINC FINGER PROTEIN 36 [Homo sapiens]
U33270	Human normal keratinocyte mRNA, clone A47, partial 3' sequence
SGC30584	Human phosphatidylinositol (4,5)bisphosphate 5-phosphatase homolog mRNA, partial cds
A004Q21	Human phosphatidylinositol (4,5)bisphosphate 5-phosphatase homolog mRNA, partial cds
WI-14374	Human phosphatidylinositol (4,5)bisphosphate 5-phosphatase homolog mRNA, partial cds
stSG1504	Human phosphatidylinositol (4,5)bisphosphate 5-phosphatase homolog mRNA, partial cds
SGC31613	MITOCHONDRIAL 2-OXOGLUTARATE/MALATE CARRIER PROTEIN
SGC31677	PIGMENT EPITHELIUM-DERIVED FACTOR PRECURSOR
WI-15114	PLATELET-ACTIVATING FACTOR ACETYLHYDROLASE 45 KD SUBUNIT
stSG4240	PLATELET-ACTIVATING FACTOR ACETYLHYDROLASE 45 KD SUBUNIT
D10656	PROTO-ONCOGENE C-CRK
Cda1ig09	REPLICATION PROTEIN A 70 KD DNA-BINDING SUBUNIT
M63488	REPLICATION PROTEIN A 70 KD DNA-BINDING SUBUNIT
SGC35513	SEX HORMONE-BINDING GLOBULIN PRECURSOR
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Between D17S849 and D17S1798 [0-6 cM]

SHGC-31105 Unidentified transcript

SHGC-31242 Unidentified transcript

SHGC-32158 Unidentified transcript

SHGC-3217 Unidentified transcript

Between D17S1798 and D17S1828 [6-9 cM]

SHGC-12572 INTEGRIN ALPHA-E PRECURSOR

SHGC-343 PLATELET-ACTIVATING FACTOR ACETYLHYDROLASE 45 KD SUBUNIT

Between D17S1828 and D17S1810 [9-11 cM]

SHGC-11989 ACETYLCHOLINE RECEPTOR PROTEIN, EPSILON CHAIN PRECURSOR

SHGC-10989 PLATELET GLYCOPROTEIN IB ALPHA CHAIN PRECURSOR

SHGC-317 Unidentified transcript

Near D17S1828 [9 cM]

SHGC-31444 Unidentified transcript

Near D17S1810 [11 cM]

SHGC-31613 MITOCHONDRIAL 2-OXOGLUTARATE/MALATE CARRIER PROTEIN

Near D17S796 [14 cM]

A004D02 Human clone 23933 mRNA sequence

A008B35 Unidentified transcript

Between D17S796 and D17S960 [14-16 cM]

WI-17813	ARACHIDONATE 12-LIPOXYGENASE
SGC35547	Highly similar to ACYL-COA DEHYDROGENASE, SHORT-CHAIN SPECIFIC PRECURSOR [Homo sapiens]
stSG1241	Highly similar to ACYL-COA DEHYDROGENASE, VERY-LONG-CHAIN SPECIFIC PRECURSOR [Rattus norvegicus]
SGC33235	Unidentified transcript
WI-13656	Unidentified transcript
WI-18574	Unidentified transcript
stSG10152	Unidentified transcript
stSG3230	Unidentified transcript
stSG4044	Unidentified transcript
stSG4773	Unidentified transcript
stSG4857	Unidentified transcript
stSG8146	Unidentified transcript

Between D17S796 and D17S786 [14-18 cM]

SHGC-166	Highly similar to DIAMINE ACETYLTRANSFERASE [Homo sapiens]
SHGC-31951	Human HepG2 3' region MboI cDNA, clone hmd3a05m3
SHGC-12725	Human cell 12-lipoxygenase mRNA, complete cds
SHGC-12899	MYOSIN HEAVY CHAIN, NONMUSCLE TYPE B
U34304	MYOSIN HEAVY CHAIN, NONMUSCLE TYPE B
SHGC-12631	RETINAL GUANYLYL CYCLASE PRECURSOR
SHGC-17245	Unidentified transcript
SHGC-31356	Unidentified transcript
SHGC-31370	Unidentified transcript
SHGC-31899	Unidentified transcript
A004I35	Unidentified transcript
A004M39	Unidentified transcript

Between D17S960 and D17S786 [16-18 cM]

stSG1426	ASIALOGLYCOPROTEIN RECEPTOR 1
WI-9178	CELLULAR TUMOR ANTIGEN P53
WI-16243	H.sapiens mRNA for RNA polymerase II largest subunit
SGC30770	Highly similar to 60S RIBOSOMAL PROTEIN L29 [Homo sapiens]
IB1796	Highly similar to ACETYLCHOLINE RECEPTOR PROTEIN, BETA CHAIN PRECURSOR [Homo sapiens]
stSG9768	Highly similar to DIAMINE ACETYLTRANSFERASE [Homo sapiens]
SGC32450	Highly similar to MYOSIN HEAVY CHAIN, NONMUSCLE TYPE B [Homo sapiens]
A002F11	Highly similar to SYNAPTOBREVIN 2 [Homo sapiens]
KIAA0050	Human mRNA for KIAA0050 gene, complete cds
WI-7423	RETINAL GUANYLYL CYCLASE PRECURSOR
SGC31498	SODIUM/POTASSIUM-TRANSPORTING ATPASE BETA-2 CHAIN
stSG77	SODIUM/POTASSIUM-TRANSPORTING ATPASE BETA-2 CHAIN
SGC31356	Unidentified transcript
SGC31370	Unidentified transcript
SGC31899	Unidentified transcript
SGC34636	Unidentified transcript
SGC34915	Unidentified transcript
WI-11341	Unidentified transcript
WI-11540	Unidentified transcript
WI-12761	Unidentified transcript
WI-14061	Unidentified transcript
WI-1410	Unidentified transcript
WI-15346	Unidentified transcript
WI-16715	Unidentified transcript
WI-18497	Unidentified transcript
WI-6216	Unidentified transcript
stSG10269	Unidentified transcript

stSG2570	Unidentified transcript
stSG294	Unidentified transcript
stSG3156	Unidentified transcript
stSG3959	Unidentified transcript
stSG8124	Unidentified transcript
stSG8669	Unidentified transcript
stSG9868	Unidentified transcript
stSG9877	Unidentified transcript

Address comments and suggestions to info@ncbi.nlm.nih.gov

List of salaried personnel:

Name:	Position:	Percent contribution to salary:
Hans M. Albertsen,	Research Instructor,	67%
Jeff Stevens,	Research associate,	100%
Ray White,	P.I., Professor,	10%